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(54) **Quantification of nucleic acid**

Quantifikation von Nukleinsäuren

Quantification d'acides nucléiques

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(56) References cited:
EP-A- 0 329 822 **WO-A-91/02815**
WO-A-91/02817

- **ABST AN MEET AM SOC MICROBIOL vol. 90, 1990, page 114 B. LAMBE ET AL. 'Quantitation of Epstein-Barr Virus (EBV) DNA in clinical specimens utilizing the polymerase chain reaction (PCR)'**
- **NUCLEIC ACIDS RESEARCH. vol. 17, no. 22, 1989, ARLINGTON, VIRGINIA US pages 9437 - 9446 M. BECKER-ANDR 'Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY)'**
- **PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, April 1990, WASHINGTON US pages 2725 - 2729 G. GILLILAND ET AL. 'Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction'**
- **NATURE. vol. 324, 13 November 1986, LONDON GB pages 163 - 166 R. K. SAIKI ET AL. 'Analysis of enzymatically amplified beta-globin and HLA-DQalpha DNA with allele-specific oligonucleotide probes'**

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The file contains technical information submitted after the application was filed and not included in this specification

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Description

[0001] The invention relates to a method for quantification of target nucleic acid in a test sample. A test kit for carrying out said method is also part of the invention.

[0002] A method for carrying out the amplification of nucleic acid in a test sample has been disclosed among others by Cetus Corp. in USP 4,683,195 and 4,683,202 the so-called polymerase chain reaction (PCR).

[0003] Recently another method for amplification of nucleic acid in a test sample, especially RNA sequences, has been disclosed in European Patent Application EP 0,329,822 by Cangene Corp.

[0004] The technique involves the transcription of multiple RNA copies from a template comprising a promoter recognized by an RNA polymerase. With these methods multiple RNA copies are transcribed from a DNA template that comprises a functional promoter recognized by the RNA polymerase. Said copies are used as a target again from which a new amount of the DNA template is obtained etc.

[0005] The process itself will not be discussed here in detail, but it concerns the so-called NASBA^{tm} technique (= nucleic acid sequence based amplification).

[0006] Amplification is an exponential process. Small differences in any of the variables which control the reaction rate will lead to dramatic differences in the yield of the amplified product. PCR as well as NASBA have wide-spread applications in genetic disease diagnosis however, these techniques only provide qualitative results.

[0007] A need exists for a method of quantifying directly, accurately, and in a reproducible manner, the amount of a specific nucleic acid present in a test sample.

[0008] A sensitive, reproducible, quantitative analysis of a test sample obtained from a patient suffering from an infectious disease, e.g. AIDS or hepatitis, can be of utmost importance in determining the extent of the infectious agent present in the patient, which information is useful in monitoring the patient treatment.

[0009] The present invention provides a method of quantifying a target nucleic acid in a test sample comprising adding a known number of molecules of a corresponding nucleic acid comprising a well-defined mutant sequence to the test sample, said mutant sequence being discriminatory from the target nucleic acid, but amplifiable with comparable efficiency, subsequently performing a transcription based nucleic acid amplification reaction of the nucleic acid, after which quantification of the amplified nucleic acid is performed by differential detection.

[0010] The target nucleic acid can be deoxyribonucleic acid (DNA) as well as ribonucleic acid (RNA).

[0011] Preferably the target nucleic acid sequence is ribonucleic acid. The differential detection necessary in this method is performed by using a probe sequence able to hybridize with both the target nucleic acid and the mutant sequence as well, or using two probes discriminating the target sequence and mutant sequence.

[0012] Said differentiation can also be performed by using a ribozyme capable of cleaving the mutant sequence, while the target sequence will not be cleaved by the ribozyme used or vice versa.

[0013] A part of the invention includes a test kit for carrying out the previously described methods.

[0014] Recently patent application WO 91/02817 was published in which a co-amplification of an internal standard nucleic acid segment and target sequence was described. The method used in this application is not a competition reaction. In contrast to the instant invention quantification in that application is performed by measuring the signals obtained and subsequently determining the ratio between both sequences amplified.

[0015] In Nuc.Ac.Res., Vol. 17, no.22, 1989, p943 7-9446, a PCR aided transcript titration assay is described by Becker Andre et al. The PATTY assay is based on the co-amplification of an in vitro generated transcript differing by a single base exchange from the target mRNA.

[0016] A method for quantitative PCR is also described by Gilliland et al in P.N.A.S. Vol. 87, 1990, 2725-2729.

[0017] In WO9102815 another quantitative amplification method is described based on PCR Again target and control differ by at least one point mutation. Detection is achieved by temperature gradient gel electrophoresis.

[0018] A method for quantifying EBV DNA using quantitative PCR is disclosed by Lambe et al. in Abst.An.Meet.Am. Chem.Soc., vol.90, p114, D206

[0019] The present invention differs significantly from that process since, among other things, competition between wild-type (target nucleic acid) and well-defined mutant sequence is an essential part of the instant invention.

[0020] The method according to the instant invention is based on the principle of competitive amplification of nucleic acid from a clinical sample containing an unknown concentration of wild-type target nucleic acid, to which has been added a known amount of a well-defined mutant sequence.

[0021] The amplification process involves:

(a) hybridizing a first primer to a first template. The first primer has a DNA sequence which is sufficiently complementary to a RNA sequence of the first template;

(b) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first template. The first DNA sequence and the first primer comprise a second template;

(c) separating the first template from the second template to allow hybridization of a second primer;

(d) hybridizing the second primer to the second template. The second primer has a DNA sequence which is sufficiently complementary to a DNA sequence of the second template. The second primer also has an antisense sequence of a promoter and an antisense sequence of a transcription initiation site for a HNA polymerase;
(e) synthesizing a second DNA sequence covalently attached to the second primer and complementary to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer. The second and third DNA sequences, the second primer and the second template comprise a third template;
(f) synthesizing a plurality of copies of the RNA sequence of the first template from the third template.

[0022] A sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3 end of the first primer is oriented towards a 3 end of the second primer on complementary strands.

[0023] This amplification process, known as NASBA, has been described in EP329822 and by Compton J., Nature 1991 Mar.7; 350 (6313): 91-92. Suitable reaction conditions are disclosed in EP329822 (for example in example 3).

[0024] Amplification of both target nucleic acid and mutant sequence as well is preferably performed with one primer set including two primers of which each primer hybridizes to the target nucleic acid and mutant sequence with the same efficiency.

[0025] This competitive amplification is performed with a fixed amount of (clinical) sample and dilution series of mutant sequence or vice versa.

[0026] The mutation in the added sequence is necessary for discriminatory detection of the wild-type and mutated amplified sequences with wild-type and mutation specific labelled oligonucleotides respectively.

[0027] This means that after competitive amplification samples are analysed in duplo using any sequence specific detection, for example:

1. gelelectrophoresis, blotting, hybridization, autoradiography, scanning;
2. Slot-blotting, hybridization, autoradiography, scanning.;
3. Non-capture bead based assay, counting; and
4. Capture bead based assay, counting.

[0028] The initial ratio of wild-type and mutated sequence will be found back in the ratio of wild-type and mutated signals. At a 1:1 ratio and equal efficiency of amplification, the reduction in signal for both wild-type and mutated sequence will be 50%. So at the dilution of mutated nucleic acid that causes a 50% reduction in signal the amount of mutated nucleic acid equals the amount of wild-type nucleic acid in the (clinical) sample.

[0029] Using a well-defined mutant sequence comprising, for instance, in the sequence a single base mutation (e. g. an A -> G transition) just one restriction enzyme, or a ribozyme, has to be used to discriminate between target nucleic acid and the mutant sequence.

[0030] Subsequently just one analysis running (for instance one gel system) is necessary in order to quantify the target nucleic acid.

[0031] Samples suitable for analysis by this method may be of human or non-human origin. The samples may be derived from cultured samples, for instance, mononuclear cells, or isolated from dissected tissue. Also blood and blood plasma, as well as brain-liquor, urine, etc. can be used as test sample material.

[0032] If, for example the test sample is blood with a target virus to be quantified according to the invention, the viral nucleic acid can be extracted from the test sample. In order to obtain a very fast, simple and reproducible procedure according to the invention the well-defined mutant sequence can be added before, during or after the target nucleic acid extraction without interference in the extraction procedure. Subsequently the competitive amplification and differential detection according to the invention can be performed directly after the extraction procedure.

[0033] Due to its high sensitivity, speed, reproducibility and accuracy, the present method can be used to quantify exactly the amount of, for instance, viruses like AIDS-virus or hepatitis virus in the test sample obtained from a patient suspected of suffering from the disease.

[0034] It can be of prime importance to know at different stages in a disease the exact amount of viruses or other disease-causing agents in order, for example, to know the dose of medication to be administered to the patient.

[0035] The test kit according to the invention is provided in its simplest embodiment with a well-defined mutant sequence and appropriate oligonucleotides viz. primers/primer pair in order to perform the desired amplification reaction and a probe sequence or ribozyme as well.

[0036] Additionally, a test kit can be supplied with the appropiate enzymes in order to carry out the amplification reaction.

[0037] The method according to the invention is illustrated by the following examples.

Example I

[0038] In vitro generated wild-type (WT) and mutant (Q) RNA were used to prove the principle of quantitative NASBA™. Plasmids used for in vitro RNA synthesis contained a 1416 bp fragment of the HIV-1 sequence resulting from a partial Fok 1 restriction enzyme digest (nucleotides 1186-2638 of the HIV-lhxb2 sequence, Ratner et al., 1987) cloned in pGEM3 or pGEM4 (Promega). The sequence between the restriction sites PstI (position 1418 on HIV-1 hxb2) and Sph I (position 1446 on HIV-1 hxb2) was changed from GAATGGGATAGAGTGCATCCAGTGCATG (OT309) in the WT to GACAGTGTAGATAGATGACAGTCGCATG (OT321) in the Q RNA. In vitro RNA was generated from these constructs with either T7 RNA polymerase or SP6 RNA polymerase. (Sambrook et al., 1989).

[0039] Reaction mixtures were treated with DNase to remove plasmid DNA. After phenol extraction and ethanol precipitation the recovered RNA was quantitated on ethidium bromide stained agarose gels by comparison to a calibration series of known amounts of ribosomal RNA. The RNA solutions were diluted to the desired concentrations and used as input for amplification by NASBA™ as described in EP 0329,822. Primers used for amplification were OT 270: (AATTCTAATACGACTCACTATAGGGGTGCTATGTCACTTCCCCTTGGTTCTCTCA , P1) and OT271 (AGTGGGGGGACATCAAGCAGCCATGCAAA, P2), generating a RNA molecule complementary to the HIV-1hxb2 sequence of 142 nt (pos 1357 to 1499). Detection of 10 µl of each amplification has been performed by electrophoresis in duplo on 3% NuSieve, 1% agarose gels (Sambrook et al., 1989) blotted onto Zeta-Probe (Biorad) using a vacuumblot apparatus (Pharmacia) and hybridized with ^{32}P labelled oligonucleotides specific for either the WT or the Q RNA sequence between above mentioned SphI and Pst 1 sites. Exposure times to X-ray films (Kodak) ranged from 30 minutes to 3 days.

[0040] Films were scanned with a LKB Ultroscan XL densitometer for quantification of the signal in the bands. Number of target molecules of both WT and Q RNA are listed in table 1.

Table 1.

Tube	Copies W. T. RNA	Copies Q RNA
1	10^3	10^1
2	10^3	10^2
3	10^3	10^3
4	10^3	10^4
5	10^3	10^5

[0041] As control amplification of WT RNA or Q RNA alone was performed. The results of the competitive NASBA™ are presented in fig. 1. At the mean of the 50% reduction for both WT and Q RNA the number of input molecules is approximately 10^3 molecules Q RNA, which equals the number of WT RNA molecules.

[0042] The formula used for determining the mean of 50% reduction for both Q and WT RNA is as follows:

$$\log(\text{conc. W.T.}) = \frac{\log([Q]\ 50\%\ \text{Sig Q}) + \log([Q]\ 50\%\ \text{Sig. WT})}{2}$$

in which ([Q] 50% Sig. Q) is the number of Q RNA molecules at which the signal using OT 321, specific for Q RNA, is only 50% of the signal obtained when Q RNA alone is amplified and ([Q] 50% Sig. WT) is the number of Q RNA molecules at which the signal using OT 309, specific for WT RNA, is only 50% of the signal obtained when WT RNA alone is amplified.

Example II

[0043] As in example 1 except input RNA molecules are as in table 2.

Table 2.

Tube	copies W.T. RNA	copies Q RNA
1	10^4	10^2
2	10^4	10^3
3	10^4	10^4

Table 2. (continued)

Tube	copies W.T. RNA	copies Q RNA
4	10^4	10^5
5	10^4	10^6

[0044] The results presented in fig. 2 show an input of 10^4 molecules of WT RNA using the formula.

$$\log.\ (\text{conc. WT}) = \frac{\log\ ([Q]\ \text{Sig Q 50\%}) + \log\ ([Q]\ \text{Sig. WT 50\%})}{2}$$

Example III

[0045] As in example 1 except that input RNA molecules are as in table 3.

Table 3.

Tube	copies W.T. RNA	copies Q RNA
1	10^5	10^3
2	10^5	10^4
3	10^5	10^5
4	10^5	10^6
5	10^5	10^7

[0046] The results presented in fig. 3 show an input of 6.5×10^4 molecules of WT RNA using the formula.

$$\log\ (\text{conc. W.T.}) = \frac{\log\ ([Q]\ \text{Sig Q 50\%}) + \log\ ([Q]\ \text{Sig. WT 50\%})}{2}$$

Example IV

[0047] Here quantitative NASBA^{tm} is applied to nucleic acid isolated from plasma of HIV-1 infected individuals. 1 ml plasma samples of 3 sero-positive HIV-1 infected individuals were used to isolate nucleic acid (Boom et al., 1990).

[0048] Nucleic acid was finally recovered in 100 µl water. Amplifications were as in example 1 except input RNA molecules were as in table 4.

Table 4.

tube	volume nucleic acid sol.	copies Q RNA
1	2 µl patient 1	10^1
2	2 µl patient 1	10^2
3	2 µl patent 1	10^3
4	2 µl patient 1	10^4
5	2 µl patient 1	10^5
6	2 µl patient 2	10^1
7	2 µl patient 2	10^2
8	2 µl patient 2	10^3
9	2 µl patient 2	10^4
10	2 µl patient 2	10^5
11	2 µl patient 3	10^1
12	2 µl patient 3	10^2

Table 4. (continued)

tube	volume nucleic acid sol.	copies Q RNA
13	2 µl patient 3	10^3
14	2 µl patient 3	10^4
15	2 µl patient 3	10^5

[0049] Results are presented in figures 4, 5 and 6 for patients 1, 2 and 3, respectively.

[0050] Results indicate the number of W.T. RNA molecules for patients 1, 2 and 3 to be 4.5×10^3, 2.1×10^3 and 1.2×10^4 in 2µl nucleic acid solution, respectively, using the formula:

$$\log (\text{conc. WT}) = \frac{\log ([Q] \text{ Sig Q } 50\%) + \log ([Q] \text{ Sig. WT } 50\%)}{2}$$

Example V

[0051] As in example 1 except that input RNA molecules are as in table 5 and that detection of NASBA amplified WT$^-$ and Q-RNA is according to the hereafter described method.

[0052] Amplified WT$^-$ and Q-RNA of 5 µl NASBA reaction was captured on streptavadin coated magnetic dynabeads (Dynal) with the biotynylated oligonucleotide OT 700 (5' Biotin-TGTTAAAAGAGACCHTCAATGAGGA 3') as intermediair. The capture hybridization process takes place at 45 °C for 30 minutes in 100 µl hybridization buffer II (5 x SSPE, 0.1% SDS, 0.1% milkpowder, 10 µg/ml denatured salm-sperm DNA; Sambrook et al., 1989). After this step the beads are washed in 2 x SSC, 0.1% BSA using a magnet to retain the beads in the reaction tube or microtiter plate.

[0053] Subsequently the RNA was hybridized with Horse Radish Peroxidase (HRP) labelled oligonucleotides specific for the WT$^-$ or Q-RNA sequence between before mentioned PstI and SphI sites, in 100 µl hybridization buffer II for 30 minutes at 45 °C.

[0054] Non-hybridized HRP-oligonucleotides are washed away using the same procedure described above. Detection of HRP retained on the beads is accomplished by addition of 100 µl substrate solution (0.45 mM TMB.HCl.H_2O, 0.5 mM CTAB, 7.65 g/l Emdex, 27 mM NaCitrate.$2H_2O$, 22.1 mM citric acid.H_2O, 2.25 mM urea-peroxid and 5.35 mM 2-chloro-acetamid).

[0055] The reaction is stopped at an appropriate time point with 50 µl 250 mM oxalic acid. The amount of substrate conversion from colorless to yellow is determined by measuring the absorbance at 450 nm in an Organon Teknika 510 microplate reader. The A_{450} values for both WT$^-$ and Q-probe are analysed as before (figure 7).

[0056] The results in figure 7 show an input of 2.7×10^2 molecules WT-RNA using the formula:

$$\log (\text{conc. WT}) = \frac{\log ([Q] \text{ Sig Q } 50\%) + \log ([Q] \text{ Sig. WT } 50\%)}{2}$$

Table 5

Tube	copies WT$^-$ RNA	copies Q-RNA
1	10^2	-
2	10^2	10^2
3	10^2	10^3
4	10^2	10^4
5	10^2	10^5
6	10^2	10^6

References:

[0057] Boom R, Sol CFA, Salimans MMM, Jansen CL, Werthiem - van Dillen PME and Van der Noordaa J. Rapid and simple method for purification of nucleic acids.
J. Clin. Microbiol. 1990; 28 : 495 - 503.

[0058] Ratner L, Fisher A, Jagodzinske HH, Mitsuya H., Lion RS, Gallo RC and Wong-Staal F. Complete nucleotide sequence of functional clones of the AIDS virus.

AIDS Res. Hum. Retroviruses 1987; 3 : 57 - 69.
[0059] Sambrook J, Maniatis T, Fritsch E.
Molecular cloning. A laboratory manual, 2nd edition. Cold Spring Harbor laboratories, Cold Spring Habor, New York, 1989.

Claims

1. A method of quantifying a target nucleic acid in a test sample comprising the steps of

   - adding a known number of nucleic acid molecules to the sample, said molecules having a nucleic acid sequence that is mutated in such a way that it can be distinguished from the target sequence during detection, but resembles the target sequence in that it comprises the same primer binding sites and is amplifiable with comparable efficiency,
   - competitively amplifying both the target sequence and the added molecules using a transcription based nucleic acid amplification method, wherein one or more primers capable of annealing to both the target sequence and the added molecules are used and, wherein at least one of said primers comprises a DNA dependent RNA-polymerase promoter sequence,
   - detecting both the target and the added, mutated sequence,
   - measuring the signals reflecting the amount of amplified target and mutated sequence respectively, and
   - calculating the amount of target sequence originally present in the sample from said signals.

2. Method according to claim 1, **characterized in that** the target nucleic acid is ribonucleic acid.

3. Method according to claim 2, **characterized in that** the added nucleic acid molecules are RNA molecules.

4. Method according to any of claim 1-3, **characterized in that** for the detection of the amplified sequences two probes are used, one comprising a sequence capable of hybridizing to the amplified RNA derived from the target sequence and not to the amplified RNA derived from the added molecules, and one probe comprising a sequence capable of hybridizing to the amplified RNA derived from the added molecules and not to amplified RNA derived from the target sequence.

5. Method according to any of claims 1-3, **characterized in that** a probe is used comprising a sequence capable of hybridizing to both the amplified RNA derived from the target sequence and the amplified RNA derived from the added molecules.

6. Method according to any of claims 1-4, **characterized in that** the sequence of the added molecules differs from the target sequence **in that** part of the sequence of the added molecules is composed of substantially the same nucleotides as the corresponding part of the target sequence, which nucleotides have been placed in a different order.

7. Method according to any of claims 1-3, **characterized in that** a ribozyme capable of cleaving the mutant sequence or target nucleic acid is used to allow the target sequence and the added molecules to be detected separately.

8. A method according to any of claims 1-7 wherein the known amount of nucleic acid molecules is added to a sample of the biological material in which the amount of the target sequence is to be determined before the sample of the biological material is subjected to a nucleic acid extraction procedure.

9. Testkit for carrying out the method of claim 1, comprising

   - a known number of nucleic acid molecules, said molecules having a nucleic acid sequence that is mutated in such a way that it can be distinguished from the target sequence during detection, but resembles the target sequence in that it comprises the same primer binding sites and is amplifiable with comparable efficiency,
   - at least one primer capable of annealing to both the target sequence and the sequence of the added molecules, comprising the sequence of a promoter recognized by a DNA-dependent RNA polymerase

Patentansprüche

1. Verfahren zum Quantifizieren einer Target-Nukleinsäure in einer Messprobe, umfassend die Schritte

   - Zugeben einer bekannten Zahl von Nukleinsäuremolekülen zur Probe, wobei die genannten Moleküle eine Nukleinsäuresequenz aufweisen, die in einer solchen Weise mutiert ist, dass sie von der Targetsequenz während der Detektion unterschieden werden kann, die aber der Targetsequenz dadurch ähnelt, dass sie die selben Primer-Bindungsstellen umfasst und mit vergleichbarer Effizienz amplifizierbar ist,
   - kompetitives Amplifizieren sowohl der Targetsequenz als auch der zugegebenen Moleküle unter Verwendung eines transkriptionsbasierten Nukleinsäure-Amplifikationsverfahrens, wobei ein oder mehrere Primer, die geeignet sind, sich sowohl an die Targetsequenz als auch an die zugegebenen Moleküle anzulagern, verwendet werden und wobei mindestens einer der genannten Primer eine DNA-abhängige RNA-Polymerase-Promotorsequenz umfasst,
   - Detektieren sowohl der Targetsequenz als auch der zugegebenen mutierten Sequenz,
   - Messen der Signale, die die Menge von amplifizierter Targetsequenz bzw. mutierter Sequenz widerspiegeln, und
   - Berechnen der Menge an Targetsequenz, die ursprünglich in der Probe vorhanden war, aus diesen Signalen.

2. Verfahren gemäss Anspruch 1, **dadurch gekennzeichnet, dass** die Target-Nukleinsäure eine Ribonukleinsäure ist.

3. Verfahren gemäss Anspruch 2, **dadurch gekennzeichnet, dass** die zugegebenen Nukleinsäuremoleküle RNA-Moleküle sind.

4. Verfahren gemäss einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** für die Detektion der amplifizierten Sequenzen zwei Sonden verwendet werden, wobei eine Sonde eine Sequenz umfasst, die geeignet ist, an die amplifizierte RNA, die von der Targetsequenz abgeleitet ist, und nicht an die amplifizierte RNA, die von den zugegebenen Molekülen abgeleitet ist, zu hybridisieren, und wobei eine Sonde eine Sequenz umfasst, die geeignet ist, an die amplifizierte RNA, die von den zugegebenen Molekülen abgeleitet ist, und nicht an amplifizierte RNA, die von der Targetsequenz abgeleitet ist, zu hybridisieren.

5. Verfahren gemäss einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** eine Sonde verwendet wird, die eine Sequenz umfasst, die geeignet ist, sowohl an die amplifizierte RNA, die von der Targetsequenz abgeleitet ist, als auch an die amplifizierte RNA, die von den zugegebenen Molekülen abgeleitet ist, zu hybridisieren.

6. Verfahren gemäss einem der Ansprüche 1 bis 4, **dadurch gekennzeichnet, dass** die Sequenz der zugegebenen Moleküle sich von der Targetsequenz dadurch unterscheidet, dass ein Teil der Sequenz der zugegebenen Moleküle aus im wesentlichen den gleichen Nukleotiden zusammengesetzt ist wie der entsprechende Teil der Targetsequenz, wobei diese Nukleotide in eine andere Reihenfolge gebracht wurden.

7. Verfahren gemäss einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** ein Ribozym, welches geeignet ist, die Mutationssequenz oder die Target-Nukleinsäure zu spalten, verwendet wird, um die getrennte Detektion der Targetsequenz und der zugegebenen Moleküle zu ermöglichen.

8. Verfahren gemäss einem der Ansprüche 1 bis 7, wobei die bekannte Menge von Nukleinsäuremolekülen zu einer Probe des biologischen Materials, in dem die Menge der Targetsequenz bestimmt werden soll, zugegeben wird, bevor die Probe des biologischen Materials einem Nukleinsäureextraktionsverfahren unterworfen wird.

9. Testkit zur Durchführung des Verfahrens des Anspruchs 1, umfassend

   - eine bekannte Zahl von Nukleinsäuremolekülen, wobei die genannten Moleküle eine Nukleinsäuresequenz aufweisen, die in einer solchen Weise mutiert ist, dass sie von der Targetsequenz während der Detektion unterschieden werden kann, aber der Targetsequenz dadurch ähnelt, dass sie die selben Primer-Bindungsstellen umfasst und mit vergleichbarer Effizienz amplifizierbar ist,
   - mindestens einen Primer, der geeignet ist, sich sowohl an die Targetsequenz als auch an die Sequenz der zugegebenen Moleküle anzulagern, umfassend die Sequenz eines Promotors, der von einer DNAabhängigen RNA-Polymerase erkannt wird.

Revendications

1. Une méthode de quantification d'un acide nucléique cible dans un échantillon à tester comprenant les étapes de :

   - addition d'un nombre connu de molécules d'acide nucléique à l'échantillon, lesdites molécules ayant une séquence d'acide nucléique qui est mutée d'une manière telle qu'elle puisse être distinguée de la séquence cible au cours de la détection, mais ressemble à la séquence cible en ce qu'elle comprend le même site de fixation d'amorces et qu'elle est amplifiable avec une efficacité comparable,
   - amplification compétitivement à la fois de la séquence cible et des molécules ajoutées en utilisant une méthode d'amplification d'acide nucléique à base de transcription, dans laquelle une ou plusieurs amorces pouvant s'anneler aussi bien à la séquence cible qu'aux molécules ajoutées sont utilisées et où au moins une desdites amorces comprend une séquence promoteur ARN polymérase dépendante de l'ADN,
   - détection à la fois de la séquence cible et de la séquence ajoutée mutée,
   - mesure des signaux reflétant la quantité de cible et de séquence mutée amplifiées respectivement, et
   - calcul de la quantité de séquence cible primitivement présente dans l'échantillon à partir desdits signaux.

2. Méthode selon la revendication 1, **caractérisée en ce que** l'acide nucléique cible est un acide ribonucléique.

3. Méthode selon la revendication 2, **caractérisée en ce que** les molécules d'acide nucléique ajoutées sont des molécules d'ARN.

4. Méthode selon une quelconque des revendications 1 à 3, **caractérisée en ce que** pour la détection de la séquence amplifiée, on utilise deux sondes, l'une comprenant une séquence pouvant s'hybrider à l'ARN amplifié dérivant de la séquence cible et non à l'ARN amplifié dérivant de molécules ajoutées et une sonde comprenant une séquence pouvant s'hybrider à l'ARN amplifié dérivé des molécules ajoutées et non à l'ARN amplifié dérivé de la séquence cible.

5. Méthode selon une quelconque des revendications 1 à 3, **caractérisée en ce qu'**on utilise une sonde comprenant une séquence pouvant s'hybrider aussi bien à l'ARN amplifié dérivé de la séquence cible qu'à l'ARN amplifié dérivé de molécules ajoutées.

6. Méthode selon une quelconque des revendications 1 à 4, **caractérisée en ce que** la séquence des molécules ajoutées diffère de la séquence cible **en ce qu'**une partie de la séquence des molécules ajoutées est composée de sensiblement les mêmes nucléotides que la partie correspondante de la séquence cible, lesdits nucléotides ayant été placés dans un ordre différent.

7. Méthode selon une quelconque des revendications 1 à 3, **caractérisée en ce qu'**on utilise un ribozyme pouvant couper la séquence mutante ou l'acide nucléique cible pour permettre la détection de la séquence cible et des molécules ajoutées séparément.

8. Une méthode selon une quelconque des revendications 1 à 7, dans laquelle la quantité connue de molécules d'acide nucléique est ajoutée à un échantillon de matériel biologique dans lequel la quantité de séquence d'acide doit être déterminée avant que l'échantillon de matériel biologique soit soumis à une procédure d'extraction d'acide nucléique.

9. Kit de test pour mettre en oeuvre le procédé selon la revendication 1 comprenant :

   - un nombre connu de molécules d'acide nucléique, lesdites molécules ayant une séquence d'acide nucléique qui est mutée de façon telle qu'elle puisse être distinguée de la séquence cible au cours de la détection, mais ressemble à la séquence cible en ce qu'elle comprend les mêmes sites de liaison d'amorce et est amplifiable avec une efficacité comparable,
   - au moins une amorce pouvant s'anneler à la fois à la séquence cible et à la séquence de molécules ajoutées comprenant la séquence d'un promoteur reconnu par une ARN polymérase dépendante d'ADN.

FIGURE 1

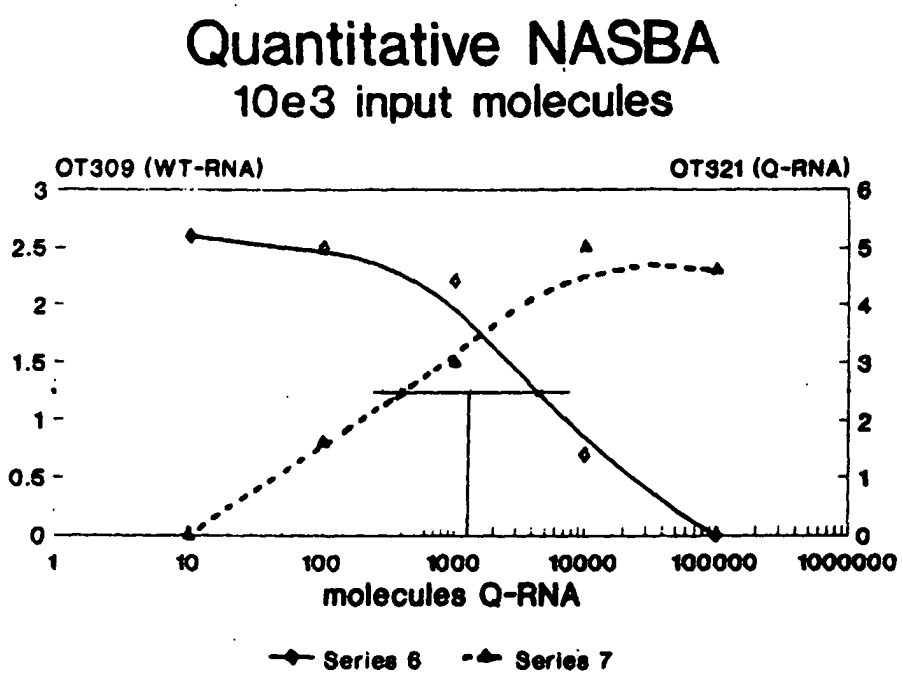


FIGURE 2

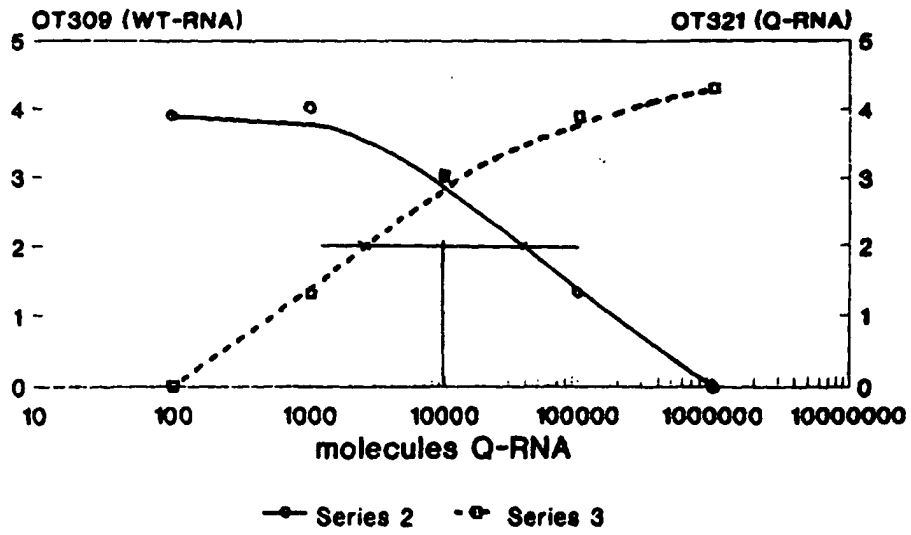


FIGURE 3

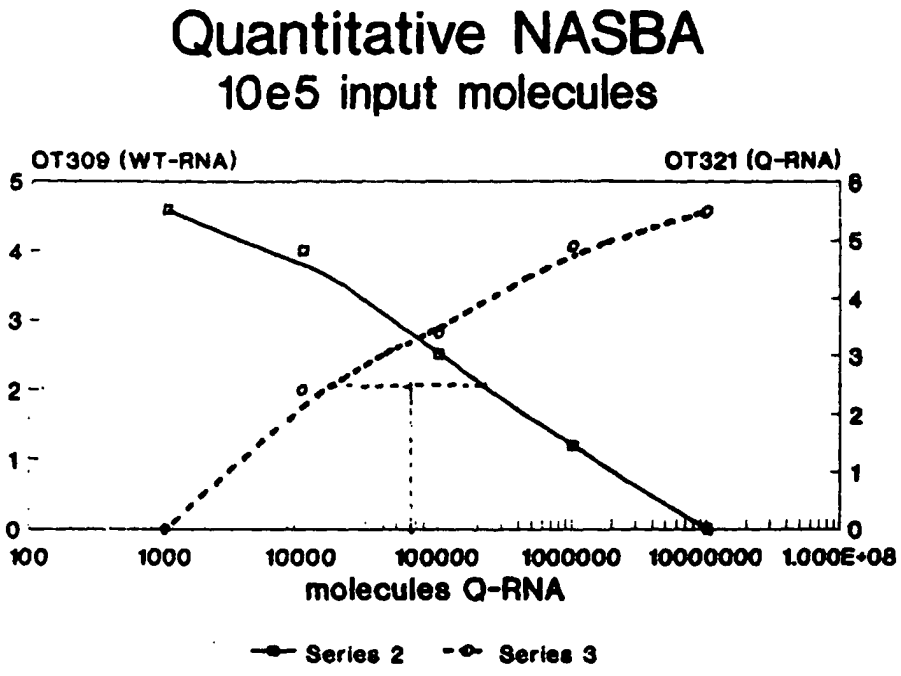


FIGURE 4

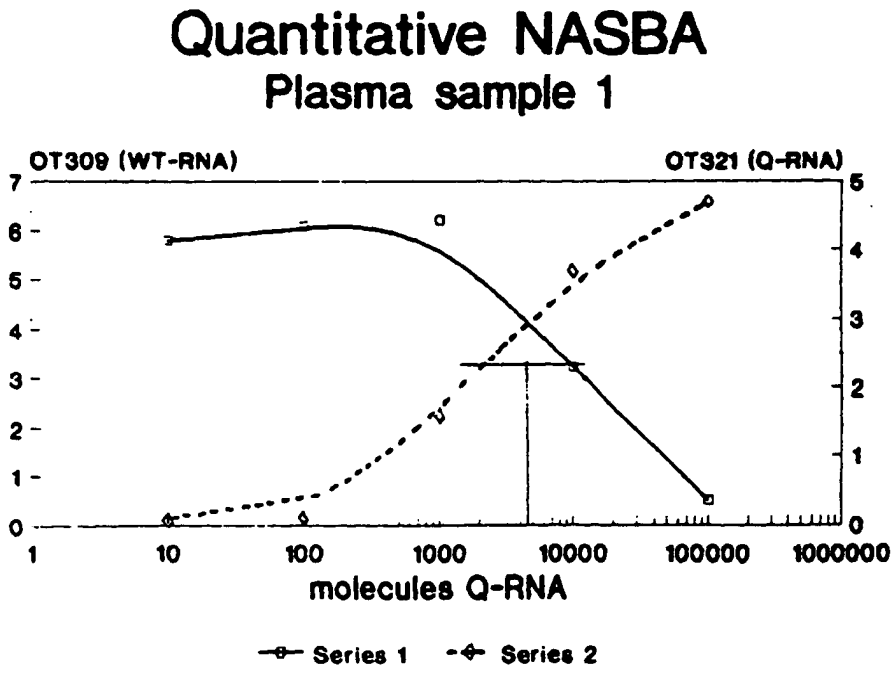


FIGURE 5

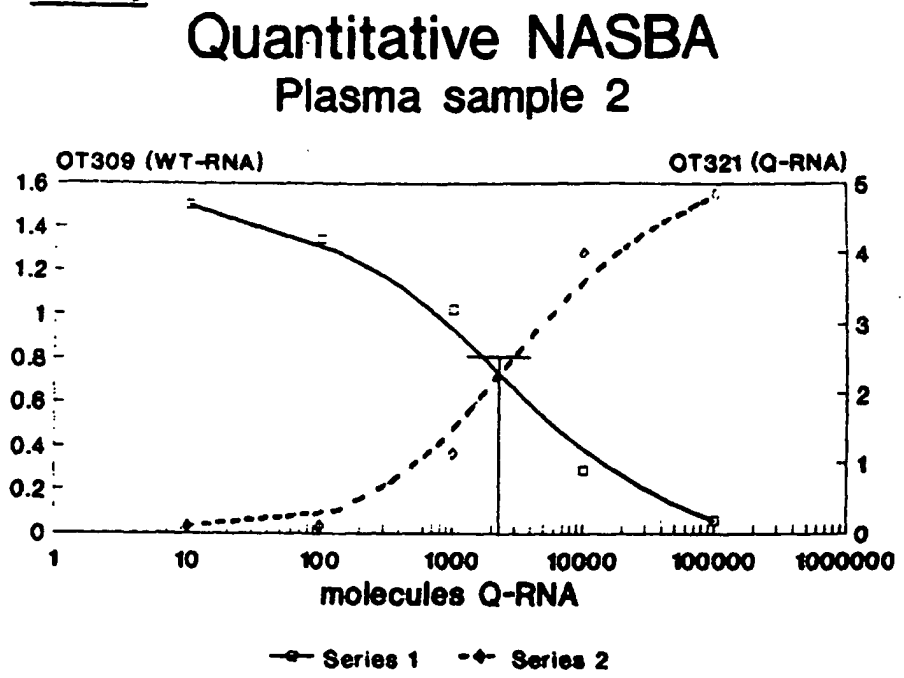
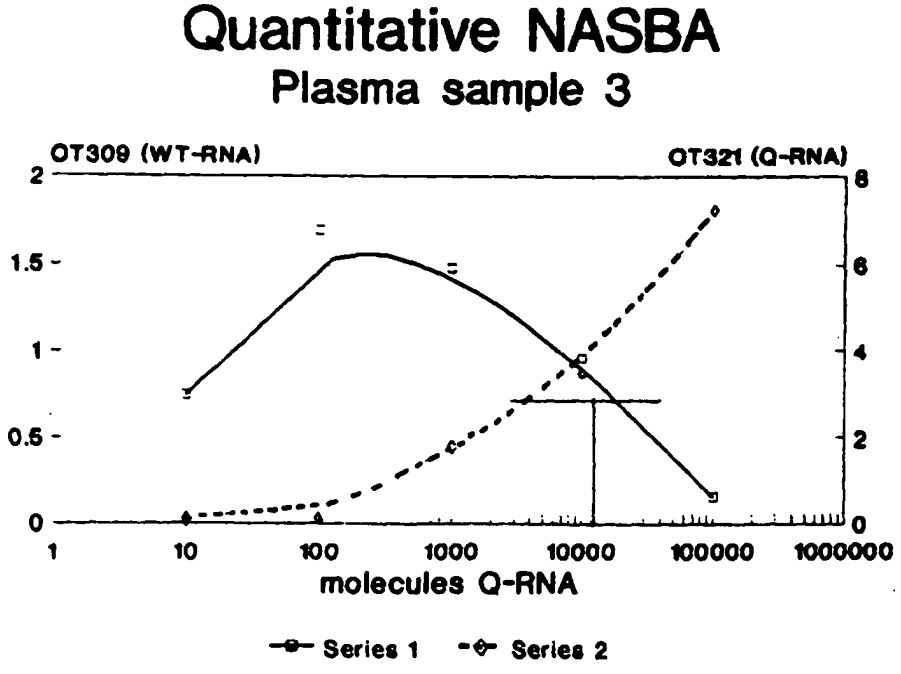


FIGURE 6



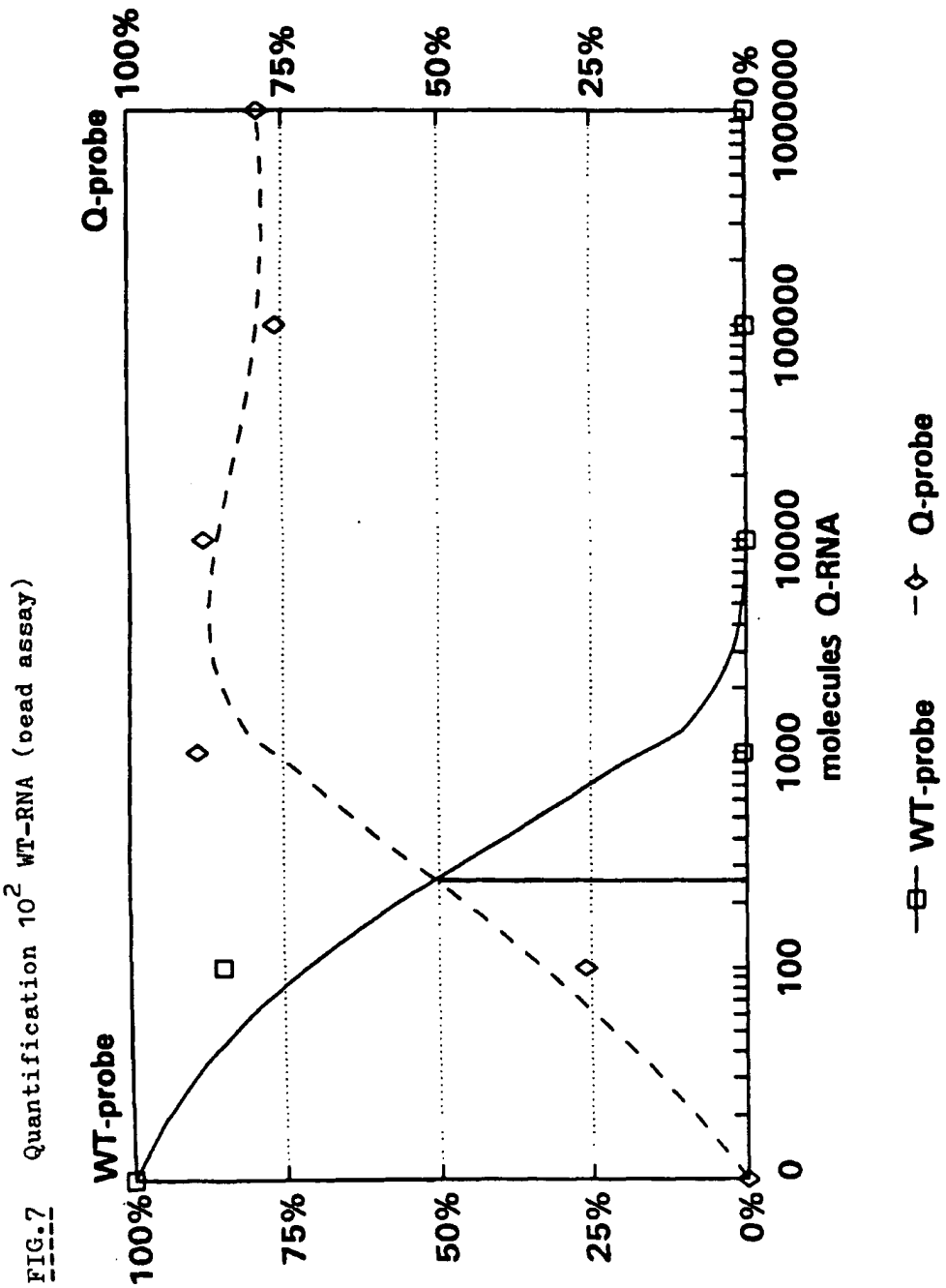


FIG.7 Quantification 10^2 WT-RNA (bead assay)